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Ro 61–1790, a New Hydrosoluble Endothelin Antagonist: General Pharmacology and Effects on Experimental Cerebral Vasospasm

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ABSTRACT

Endothelin (ET) receptor antagonists are of great potential clinical interest for the treatment pathological conditions associated with vasospasm, such as subarachnoid hemorrhage (SAH). We developed for parenteral use a compound of a class of trifunctionalized heteroarylsulfonamide pyrimidines specially designed for high water solubility. Ro 61–1790 [5-methyl-pyridine-2-sulfonic acid 6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2-(2–1H-tetrazol-5-yl-pyridin-4-yl)-pyrimidin-4-ylamide] is a competitive ET antagonist with an affinity to ET_A receptor in the subnanomolar range. It has a ~1000-fold selectivity for the ET_A *vs.* the ET_B receptor as assessed on functional assays (*e.g.*, ET-1-induced inositol-1,4,5-triphosphate release or ET-1-induced intracellular calcium mobilization). Ro 61–1790 also had a high functional potency for inhibiting contraction induced by ET-1 on isolated rat aorta (ET_A receptors; pA_2 = 9.5) or by sarafotoxin S6c on rat trachea (ET_B receptors; pA_2 = 6.4). *In vivo*, Ro 61–1790 inhibited the pressor effect of big ET-1 in pithed rats with an ID_{50} value of 0.05 mg/kg. Intravenous bolus of Ro 61–1790 induced a long-lasting antihypertensive effect in deoxycorticosterone acetate salt rats instrumented with telemetry. In a double-hemorrhage canine model of SAH, Ro 61–1790 both prevented and reversed cerebral vasospasm in a dose-dependent manner. In an established cerebral vas - spasm, 3 mg/kg Ro 61–1790 i.v. was half as efficacious as intrabasilar papaverine. Ro 61–1790 (20 mg/kg/day) totally prevented the occurrence of vasospasm. In summary, these data demonstrate that Ro 61–1790 is a potent and selectiv ET_A receptor antagonist suitable for parenteral use and potentially useful for preventing delayed ischemic deficit in patients with SAH.

ET has been shown to play a role in a variety of diseases such as congestive heart failure and, more recently, in cerebral vasospasm after SAH (Kiowski *et al.*, 1995; Roux *et al.*, 1995; Shigeno *et al.*, 1995; Zimmermann *et al.*, 1996; Zuccarello *et al.*, 1996). Cerebral vasospasm remains one of the major causes of mortality and morbidity after SAH (Dorsch and King, 1994). An agent capable of preventing the progressive delayed ischemic deficit due to vasospasm without altering blood pressure would represent a true improvement over the current treatment with calcium channel blockers that lack cerebrovascular selectivity. Unfortunately, most ET antagonists tested in animal models of SAH were active only by intracisternal administration or would lack sufficient water solubility to be repeatedly injected intravenously.

Ro 61–1790 [5-methyl-pyridine-2-sulfonic acid 6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2-(2–1H-tetrazol-5-yl-pyridin-4-yl)-pyrimidin-4-ylamide disodium salt] is a follow-up compound from bosentan, the first ET receptor antagonist used in clinical trials (Clozel *et al.*, 1994). Ro 61–1790 was optimized for a high aqueous solubility and high ET_A potency as well as its effects in a canine model of SAH.

In the present report, we evaluated the general pharmacological characteristics of Ro 61–1790 and its effects on cerebral vasospasm. We show that Ro 61–1790 is one of the most potent nonpeptide ET_A receptor antagonists ever identified that is able to reverse cerebral vasospasm in an experimental model of SAH.

Methods

Cell Culture

Rat aortic endothelial, rat mesangial, human smooth muscle, CHO and baculovirus-infected insect cells (Sf9 cells) and COS-1 cells expressing recombinant human ET_A and ET_B receptor were cultured as described previously (Breu *et al.*, 1995; Clozel *et al.*, 1994).

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ABBREVIATIONS: ET, endothelin; SAH, subarachnoid hemorrhage; CHO, Chinese hamster ovary; COS, African green monkey kidney; BSA, bovine serum albumin; CSF, cerebrospinal fluid; DOCA, deoxicorticosterone acetate; HEK, human embryonic kidney; HPTLC, high-performance thin-layer chromatography; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; i.v., intravenous.

Preparation of Membranes

Microsomal membranes were prepared as described previously (Breu *et al.*, 1993). Cells expressing recombinant ET_A or ET_B receptor were broken by three freeze/thawing cycles in hypotonic Tris buffer (5 mM Tris, pH 7.4, 1 mM $MgCl_2$), resuspended in the same buffer with 250 mM sucrose and stored in aliquots at −80°C. Other tissues were homogenized in 5 mM Tris buffer, pH 7.4, containing 1 mM $MgCl_2$ and 250 mM sucrose with a Polytron (Kinematica, Littau-Luzern, Switzerland) and subsequently with a potter homogenizer (Vetter, Bender U. Hobeim, Zürich, Switzerland). After centrifugation at 3000 × *g* for 15 min at 4°C, the supernatant was centrifuged again at 72,000 × *g* for 40 min. The resulting pellet was finally suspended in 2.5 ml of 75 mM Tris buffer, pH 7.4, containing 25 mM $MgCl_2$ and 250 mM sucrose and stored frozen at −80°C. Protein content was determined using BSA as a standard.

Binding Assay

Competition binding assays on ET_A receptors were performed on membrane preparations of baculovirus-infected insect cells and COS-1 cells expressing human recombinant ET_A receptor or on attached cells, such as CHO cells, human smooth muscle cells and rat mesangial cells, using [^{125}I]ET-1 as radiolabeled ligand. Binding assays on ET_B receptors were also performed in the presence of [^{125}I]ET-1 using membranes of COS-1 cells expressing human recombinant ET_B receptor or membranes of human placenta. Membranes of porcine or rat trachea were used for binding studies on ET_B receptors mediating constriction. Because rat and porcine trachea contain both ET_A and ET_B receptors, the ET_B-selective agonist [^{125}I]sarafotoxin S6c was used as labeled ligand in these experiments. Suspensions of microsomal membranes were defrosted and centrifuged at 25,000 × *g* for 10 min. The pellet was resuspended at 22°C in 50 mM Tris buffer [pH 7.4, 25 mM $MnCl_2$, 1 mM EDTA and 0.5% (w/v) BSA]. Then, 50 μl of this suspension containing 0.1 to 30 μg of protein was used in a 250-μl assay containing the same buffer with 32 pM [^{125}I]ET-1 or [^{125}I]sarafotoxin S6c and increasing amounts of unlabeled Ro 61–1790. After a 2- to 3-hr incubation at 22°C, bound and free ligands were separated by filtration. Binding assays with whole attached cells were performed in 500 μl of Dulbecco's modified Eagle's medium containing 2 mg/ml BSA and 25 mM HEPES. After incubation (2 hr, 22°C) in the presence of 35 pM [^{125}I]ET-1 and increasing concentrations of Ro 61–1790, the cells were extensively washed and finally solubilized in 1% (w/v) sodium dodecyl sulfate, 0.5 M NaOH and 100 mM EDTA. Each assay was performed three times in triplicate, and nonspecific binding was assessed in the presence of 100 nM unlabeled ET-1. Specific binding was defined as the difference between total binding and nonspecific binding. IC_{50} values were determined after logit/log transformation of the binding data. Inhibitory constants (K_i values) were calculated from IC_{50} curves using the Cheng-Prusoff equation. The Hill coefficients were taken as the slope of plots of log [percent bound/(100 − percent bound)] *vs.* log concentration of Ro 61–1790.

Specificity

The specificity of Ro 61–1790 as an ET receptor antagonist was assessed by measuring the ability of Ro 61–1790 to compete with receptor-specific ligands in 53 different ligand binding assays. Ro 61–1790 was tested at 1 μM in duplicate. These assays were performed by Quintiles (Edinburgh, UK) and Cerep (Celle l'Evescault, France).

In Vitro Functional Inhibitory Potency

Inositol phosphate formation. The ET-1-mediated formation of IP_3 was assessed on COS-1 cells expressing recombinant human ET_A or ET_B receptor through a modified method of Berridge *et al.* (1983). Briefly, the cells were cultured for 2 days in 24-well plates in the presence of phosphoramidon, incubated for 24 hr with 1 μCi/ml *myo*-[^{3}H]inositol, washed three times with phosphate-buffered saline containing 10 mM LiCl, incubated in the same medium for 30 min and finally incubated for 30 min at 37°C with 10 nM ET-1 in presence of varying concentrations of Ro 61–1790. Incubation was terminated by 7.5% (w/v) trichloroacetic acid. Separation of [^{3}H]inositol phosphates was carried out by ion exchange chromatography on BioRad (Hercules, CA) AG 1-X8 columns using serial elution with increasing concentrations of formate. Finally, radioactivity was measured by liquid scintillation counting.

Ca^{++} mobilization. HEK 293 cells were cotransfected with the plasmid pCEP4/Aq, which contains the cDNA coding for aequorea victoria aequorin together with plasmids expressing human ET receptors using Lipofectamin (Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. After 48 hr, the cells were trypsinized, washed and incubated in Dulbecco's modified Eagle's growth medium supplemented with 0.1% fetal calf serum and 10 μM Coelenterazine (Molecular Probes, Eugene, OR) for 4 hr at 37°C in an incubator (Button and Brownstein, 1993). Then, the cells were washed in phosphate-buffered saline and resuspended in ECB2 buffer composed of (in mM) NaCl 140, KCl 20, HEPES 20, glucose 5, $CaCl_2$ 2 and BSA 0.1 mg/ml, pH 7.4, at a density of 10^6 cells/ml. Ninety microliters of these cells were incubated with 30 μl of inhibitor diluted in ECB2 for 30 min at 4°C. The light production was triggered by the injection of 30 μl of ET diluted in phosphate-buffered saline and measured in a Luminoskan (Bioconcept, Allschwil, Switzerland) for 8 sec.

Isolated rat aortic rings and trachea contraction. Male 14–16-week-old Wistar-Kyoto rats were anesthetized with Inactin (sodium thiobutabarbital, 100 mg/kg intraperitoneally), the chest was opened and the thoracic aorta was removed and cut into 5-mm rings. The endothelium was removed by gentle rubbing of the intimal surface, and each ring was suspended in a 10-ml isolated organ chamber containing gassed (95% O_2/5% CO_2) and warmed (37°C) Krebs-Henseleit solution composed (of mM) NaCl 115, KCl 4.7, $MgSO_4$ 1.2, $KHPO_4$ 1.5, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 10. Isometric force was recorded. The rings were stretched to a resting force of 3 × *g*. After a 60-min equilibration period, the rings were contracted using norepinephrine (10^{-7} M). Endothelium denudation was assessed by the absence of relaxation to acetylcholine (10^{-5} M). The rings were then washed and stretched if necessary until a stable base-line force was obtained. The rings were incubated with various concentrations (3×10^{-7} to 3×10^{-6} M) of Ro 61–1790. After 10 min, cumulative doses of ET-1 were added, and the interval between doses was determined by the time required for the force generated to reach a plateau. For the tracheal rings, the trachea was removed and cut into 5-mm rings. The epithelium was removed by gentle rubbing of the luminal surface, and each ring was suspended in a 10-ml isolated organ chamber containing gassed and warmed Krebs-Henseleit solution as described above. The rings were stretched to a resting force of 2 × *g*. After a 60-min equilibration period, the rings were contracted using potassium chloride (50 mM). The rings were then washed and stretched if necessary until a stable base-line force was obtained. After a 10-min incubation with Ro 61–1790 (10^{-6} to 10^{-5} M), cumulative doses of sarafotoxin S6c were added. The interval between doses was determined by the time required for the force generated to reach a plateau.

The maximum force was defined as the force generated with the highest concentration yielding a maximal effect, and from this the ET-1 or sarafotoxin S6c concentration was calculated yielding a half-maximal effect (EC_{50}). Contractile and relaxant responses are expressed as a percentage of the maximal response. The pA_2 value (negative logarithm of the molar concentration of antagonist that causes a 2-fold parallel shift to the right of the agonist concentration-response curve) was determined as described elsewhere (Clozel *et al.*, 1994).

In Vivo Functional Inhibitory Potency

Male Wistar rats (340–360 g; BRL, Füllinsdorf, Switzerland) were anesthetized with sodium hexobarbital (150 mg/kg Evipan intraperi-

toneally). After tracheal intubation, the rats were pithed with a steel rod and artificially ventilated with room air using a rodent ventilator (model 683; Harvard Apparatus, South Natick, MA) at a tidal volume of 2 ml and a rate of 65 strokes/min. The animals were kept warm at 38°C. The femoral artery and vein were cannulated for blood pressure measurement and intravenous injection of drugs, respectively. After stabilization of blood pressure, various doses of Ro 61–1790 or saline (1 ml/kg) were injected. At 5 min later, the first dose of big ET-1 (the inactive precursor of ET-1) or ET-1 itself was injected intravenously in a volume of 0.5 ml/kg. Increasing doses were injected in a cumulative manner, with each dose given after stabilization of the effect of the previous dose on blood pressure.

In Vivo Effect in Conscious Normotensive and Hypertensive Rats

Wistar rats (300 g) were instrumented with a telemetry system as described previously, and some of them ($n = 6$) made hypertensive by implantation of DOCA pellets as described previously (Brockway *et al.*, 1991; Karam *et al.*, 1996). Briefly, rats were anaesthetized with an intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine. After a flank incision, the right kidney was removed, the pressure transducer was inserted into the abdominal aorta, the transmitter body was secured to the inner surface of the peritoneal wall and the 40-mg DOCA pellet was implanted subcutaneously and renewed every 2 weeks. Rats received drinking water containing 1% NaCl. With this procedure, within 4 weeks the rats develop severe hypertension (Karam *et al.*, 1996). After having received an intravenous bolus dose of 0.3 to 10 mg/kg Ro 61–1790, rats were monitored for 24 hr for heart rate and arterial blood pressure.

Effects in a Dog Model of SAH

Vasospasm of the basilar artery of beagle dogs was induced as described previously (Roux *et al.*, 1995). Briefly, mongrel dogs (10–12 kg; BRL, Füllinsdorf, Switzerland) were anesthetized with 20 mg/kg thiamylal sodium i.v. (Surital; Parke-Davis, Berlin, Germany). The trachea of the dogs was intubated, and the lungs were ventilated in the presence of 1% isoflurane. The cisterna magna was punctured with a 22-gauge spinal needle; 4 ml CSF was withdrawn, and fresh blood (0.5 ml/kg) was slowly injected into the subarachnoid space. The same procedure was repeated on day 2. On day 4, the dogs were anaesthetized with 30 mg/kg pentobarbital intravenously and ventilated. Tidal volume was 10 to 15 ml/kg, and the respiration rate was 12 to 15/min. End-tidal Pco_2 was continuously monitored with a Datex Normocap (Helsinki, Finland). A Millar 5F pressure transducer was inserted into the left femoral artery for arterial blood pressure and heart rate monitoring. All dogs received a bolus of 50 U/kg heparin (Liquemin; F. Hoffmann-La Roche, Basel, Switzerland). A Fastrack-10 (Target Therapeutics, Fremont, CA) was inserted into the spinal artery through the right vertebral artery, and its tip was placed at the basilar rhomboid level. A bolus of 1 ml Iopamiro 370 (Bracco, Milan, Italy) was necessary to visualize the basilar artery. Blood pressure and heart rate were continuously recorded on a Mark XII chart recorder (Western GraphTec, Irvine, CA). Angiograms of the basilar artery was analyzed by a special digitizing system (Cardio 500; Kontron Bildanalyse, Munich, Germany) as previously described (Roux *et al.*, 1995).

Reversal of established vasospasm. On day 4 after SAH, the dogs were randomly assigned to receive either placebo (saline, $n = 5$) or Ro 61–1790: 0.3, 1 or 3 mg/kg as a slow bolus followed by an infusion of the same dose per hour over 2 hr ($n = 5$, 6 or 6, respectively). Angiograms of the basilar artery were performed every 30 min after treatment over 2 hr. Before the dogs were killed, two consecutive last angiograms were performed after papaverine (100 mg infused locally) was administered to maximally vasodilate the basilar artery (Macdonald *et al.*, 1995), and a CSF sample was withdrawn to measure the concentration of Ro 61–1790.

A separate group of dogs received either an intravenous bolus of 10 mg/kg Ro 61–1790 without additional treatment ($n = 6$) or 1 mg/kg Ro 61–1790 as a short hyperselective intravertebrobasilar infusion via the Fastrack-10 catheter ($n = 3$). Before a final angiogram, all dogs received a local infusion of papaverine as described above. Results are expressed as percentage of papaverine effect set at 100%.

Prevention of vasospasm. Determination of the minimal effective dose of Ro 61–1790 that prevented the occurrence of vasospasm required additional experiments. Thirty dogs were blindly randomized 4 hr after the first intracisternal blood injection to receive either placebo (1 ml saline i.v. bolus b.i.d.; $n = 7$), 4 or 10 mg/kg i.v. Ro 61–1790 b.i.d. ($n = 11$ and 12, respectively). The doses were calculated to obtain an area under the curves of 50 and 150 μg·hr/ml for the low and high dose, respectively. On day 4, angiograms were performed as described above ≥4 hr after the last drug administration. A second angiogram was performed after intrabasilar injection of papaverine as described above. Results are expressed as cross-sectional area (mm^2). Plasma and CSF concentrations of ET-1 were determined on days 0 (pre-SAH), 2 and 4 and measured according to a method previously described (Löffler and Jacot-Guillarmod; 1992).

Single-Dose Pharmacokinetics

Single-dose pharmacokinetic studies with Ro 61–1790 have been performed in rats, dogs, rabbits, cynomolgus and rhesus monkeys after intravenous bolus/infusion at the 5–10 to 60 mg/kg dose level. Ro 61–1790 was quantified in plasma using HPTLC after protein precipitation. Analysis was carried out on HPTLC silica 60F254 (20 × 10 cm) plates using an eluent consisting of ethylacetate/methanol/water/diethylamine (70:10:20:15, v/v/v/v). Post-chromatographic fluorescence enhancement was done by immersion into Triton X-100 in chloroform and *n*-hexane before *in situ* fluorescence (λ_{exc} = 313 nm, $\lambda_{em} > 400$ nm) by means of a thin-layer chromatographic scanner. Quantification was based on external standards using peak height. The quantification limit of the HPTLC assay was determined to be 0.1 μg Ro 61–1790/ml plasma.

Expression of Results

Results are expressed as mean ± S.E.M. unless otherwise specified. Groups of dogs with SAH were compared using analysis of variance. Individual comparisons of treatment groups *vs.* placebo were obtained with a two-tailed Dunnett's analysis of variance. A value $P < .05$ was considered significant.

Drugs

[^{125}I]ET-1, [^{125}I]ET-3 and [^{125}I-His]sarafotoxin S6c were obtained from Anawa Trading S.A. (Wangen, Switzerland). *myo*-[^{3}H]Inositol was from Amersham Rahn (Zürich, Switzerland). ET-1, big ET-1 and sarafotoxin S6c were obtained from Peninsula Laboratories (Merseyside, UK). They were dissolved in methanol/water (50:50) for *in vitro* studies or saline plus 0.1% BSA for *in vivo* studies. Dilutions were always performed in solutions containing 0.1% BSA. Ro 61–1790 was synthesized at F. Hoffmann-La Roche Ltd. The corresponding disodium salt was obtained by modification of the parent compound with sodium methylate in tetrahydrofuran as a solvent. For *in vitro* studies, Ro 61–1790 (disodium salt or parent compound) was dissolved in water. Norepinephrine hydrochloride, lithium chloride and potassium chloride were from Fluka Chemical, and acetylcholine hydrochloride was from Sigma Chemical (St. Louis, MO).

Results

Structural and Physicochemical Characteristics

Ro 61–1790 (molecular weight, 577.6) belongs to a class of trifunctionalized heteroarylsulfonamido pyrimidines (Breu *et al.*, 1996) and was developed by implementing further functional groups into the bosentan scaffold with the aim to enhance binding affinity and aqueous solubility (fig. 1). The molecule is characterized by a methylpyridylsulfonamido

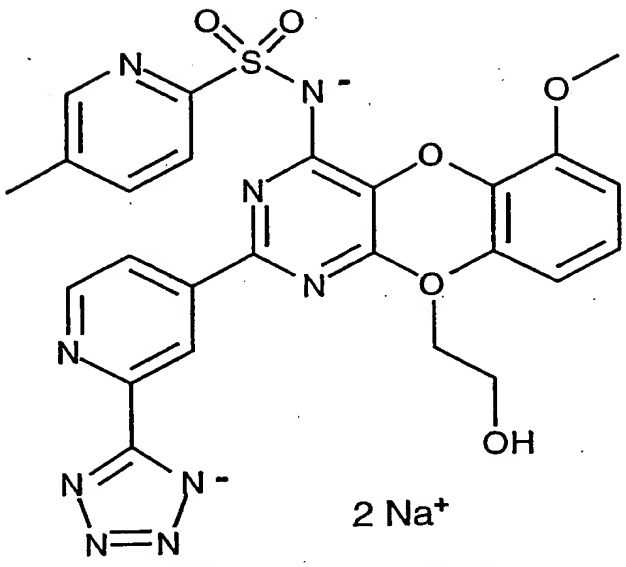

Fig. 1. Structure of Ro 61–1790 disodium salt.

group and a tetrazolylpyridinyl substituent attached to the central pyrimidine template in positions 4 and 2, respectively, which resulted into considerably improved binding affinity for the ET_A receptor in comparison with bosentan. Due to the methylpyridylsulfonamido and tetrazole functional groups, Ro 61–1790 is a weak diacid with pK_a values of 4.5 and 3.3, respectively. Ro 61–1790 has a high aqueous solubility (25% at physiological pH values), which makes it particularly suited for parenteral use.

Binding Assay and Selectivity

Affinity of Ro 61–1790 for the ET receptors was assessed in different cells and tissues (table 1). Ro 61–1790 effectively inhibited specific [^{125}I] ET-1 binding to ET_A receptors with an affinity of 0.1 to 1.7 nM (table 1). Ro 61–1790 was less effective in inhibiting specific binding of [^{125}I] labeled ET-1, ET-3 or sarafotoxin S6c to ET_B receptor. Except for the binding of [^{125}I] ET-3 on endothelial cells, the corresponding Hill coefficients (n_H) were in all cases close to unity that indicated a competitive interaction on a single population of binding sites. For endothelial cells, we performed a LIGAND evaluation of all individual curves that did not reveal additional binding sites.

To determine its specificity for the ET receptors, the inhibitory activity of Ro 61–1790 was tested in >50 receptor assays. At a concentration of 1 μM, Ro 61–1790 did not inhibit the binding of any of the ligand tested (<20% inhibition observed in a peripheral benzodiazepine receptor type). Thus, Ro 61–1790 displays high specificity for ET receptors and shows a high affinity for the ET_A receptor.

In Vitro Functional Inhibition

High potency of Ro 61–1790 on ET_A binding assay was associated with a high potency in functional assays. In rat aortic rings (ET_A receptors), Ro 61–1790 produced concentration-dependent, parallel rightward shifts in the ET-1 dose-response curve without any significant changes in the maximal responses and with a pA_2 from the Schild analysis of 9.5 (fig. 2A). Maximal force generated with ET-1 was 63 ± 3, 53 ± 6, 60 ± 1 and 58 ± 2 mN for saline and increasing concentrations of Ro 61–1790, respectively. The Schild analysis yielded a slope not different from unity, suggesting that Ro 61–1790 behaves as a competitive antagonist. Ro 61–1790 inhibited the constricting effect of sarafotoxin S6c (ET_B receptors on rat trachea) also in a competitive manner, with a pA_2 of 6.4 (fig. 2B). Maximal force generated with sarafotoxin S6c (expressed in percent KCl contraction) was 83 ± 9%, 118 ± 15%, 113 ± 13% and 110 ± 3% for saline and increasing concentrations of Ro 61–1790, respectively.

Ro 61–1790 was devoid of any agonist activity. Thus, Ro 61–1790 was 1000-fold more selective for inhibiting the ET_A- than the ET_B-mediated contraction. Similarly, a ~1000 fold selectivity for ET_A receptors was observed for ET-1-induced release of 1,4,5-IP_3 by ET_A- or ET_B-expressing COS-1 cells. The ET-1-mediated release of 1,4,5-IP_3 was inhibited by Ro 61–1790 with IC_{50} values of 5 nM and 3 μM for ET_A and ET_B receptors, respectively (fig. 3A).

This high selectivity of Ro 61–1790 for ET_A receptors was also observed when intracellular calcium mobilization was measured in HEK 293 cells that were transiently transfected with recombinant human ET_A or ET_B receptors. The ET-1-mediated calcium release was inhibited by Ro 61–1790 in a concentration-dependent manner, and the IC_{50} values were 0.23 ± 0.1 and 422 ± 450 nM for ET_A and ET_B receptors, respectively (fig. 3B).

In Vivo Inhibition of Big ET-1 and ET-1 Effects

The *in vivo* pharmacological effects of Ro 61–1790 were examined in anesthetized pithed rats challenged with increasing doses of big ET-1. After bolus administration, Ro 61–1790 alone was devoid of effect on blood pressure in

TABLE 1
Binding characterization of Ro 61-1790 on different ET receptor subtypes

Receptor subtype	Cell or tissue	^{125}I-Labeled ligand	K_i	Hill coefficient
			nM	
ET_A	Rec. Sf9	ET-1	1.7 ± 0.3	1.1
	Rec. COS	ET-1	0.6 ± 0.3	1.1
	Rec. CHO	ET-1	0.13 ± 0.03	1.2
ET_B	Rec. COS	ET-1	1930 ± 340	0.9
	Placenta (human)	ET-1	175 ± 56	0.8
	Endothelial cells (rat)	ET-3	36 ± 14	0.7
	Tracheal SMCs (rat)	ET-3	98 ± 67	0.9
	Trachea (porcine)	SRTX S6c	215 ± 68	1.1

Competition binding assays with the indicated radioligands were performed on whole CHO cells, rat endothelial cells and rat tracheal smooth muscle cells (SMCs). The other tissues were tested as membrane preparation. Data were obtained in triplicate from at least three independent experiments. Rec, recombinant.

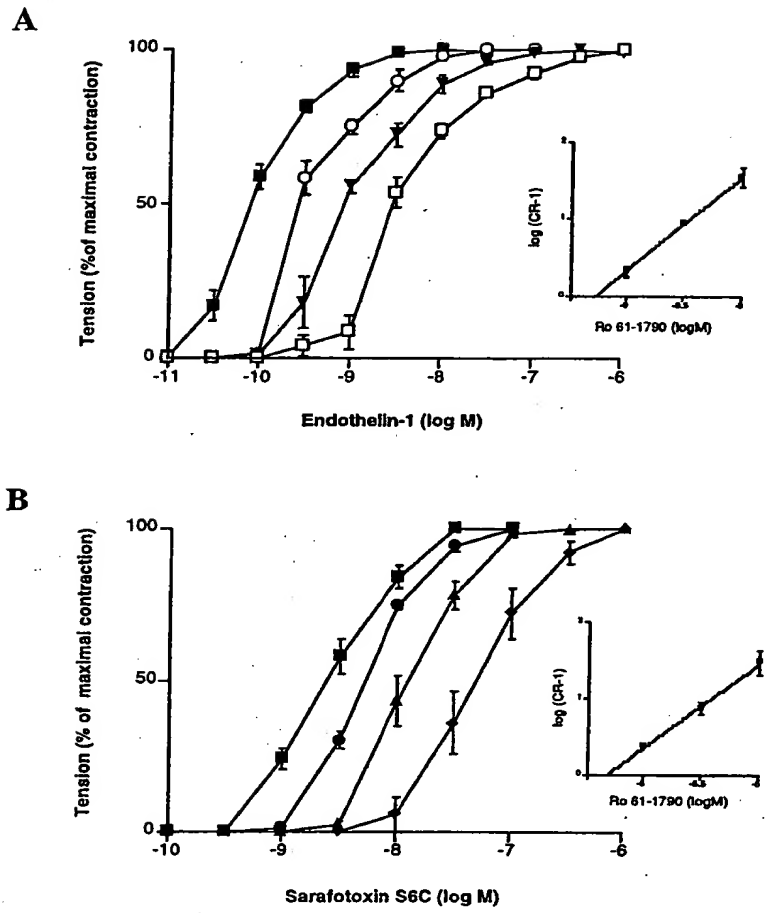

Fig. 2. Effect of Ro 61–1790 (○, 10^{-9}; ▼, 3×10^{-9}; □, 10^{-8}; ●, 10^{-8}; ▲, 3×10^{-8}; ◆, 10^{-5} M) and saline (■) on the concentration-response curve of (A) ET-1-mediated contraction of isolated rat aorta without endothelium and (B) sarafotoxin S6c-mediated contraction of isolated rat trachea without epithelium. Inset, Schild analysis for inhibition of ET-1 contraction in rat isolated aorta or sarafotoxin S6c in rat trachea. Results are represented as the mean ± S.E.M. of experiments performed in triplicate.

pithed rats. Ro 61–1790 dose-dependently inhibited the pressor effect of increasing doses of big ET-1 with an ID_{50} of 0.05 mg/kg (fig. 4). Ro 61–1790 also dose-dependently inhibited the pressor effects of ET-1 in pithed rats with an ID_{50} of 0.06 mg/kg. The transient depressor effect of ET-1 (blood pressure lowering) was inhibited by Ro 61–1790 with an ID_{50} of >10 mg/kg.

Effects in Normotensive and DOCA-Salt Hypertensive Conscious Rats

Ro 61–1790 up to an i.v. bolus dose of 10 mg/kg induced no effect on mean arterial pressure in normotensive rats (data not shown). All DOCA-salt rats had a base-line mean arterial pressure of >150 mm Hg. Ro 61–1790 dose-dependently decreased mean arterial pressure in DOCA-salt rats with a minimal efficacious dose of 1 mg/kg i.v. At 3 mg/kg, Ro 61–1790 showed a prolonged antihypertensive effect lasting >12 hr despite a terminal half-life of 0.8 hr (fig. 5). At 8 hr after intravenous administration of 1, 3 and 10 mg/kg i.v. bolus, Ro 61–1790 showed a maximal decrease of MAP of −20, −25 and −29 mm Hg, respectively ($P < .001$) (fig. 5). Ro 61–1790 did not induce reactive tachycardia. At 6 hr after treatment, heart rate was 309 ± 6, 314 ± 9, 319 ± 9, 341 ± 9 and 314 ± 5 beats/min in the control and 0.3, 1, 3 and 10 mg/kg Ro 61–1790 groups, respectively.

Effects of Ro 61–1790 in Dogs with SAH

Reversal of established cerebral vasospasm. Before treatment, SAH induced an average 65 ± 1% decrease of the cross-sectional area of the basilar artery that was not significantly different among treatment groups. At 30 min after the intravenous bolus administration, Ro 61–1790 showed a dose-dependent reversal of basilar artery vasospasm with a diameter increase of 0.01 ± 0.01, 0.03 ± 0.02, 0.06 ± 0.03, 0.09 ± 0.01 and 0.09 ± 0.02 mm in the placebo and 0.3, 1, 3 and 10 mg/kg groups, respectively. Ro 61–1790 at a dose of 10 mg/kg did not alter arterial blood pressure up to 2 hr after bolus administration. At 2 hr after infusion, 3 mg/kg/hr Ro 61–1790 increased the cross-sectional area by 47 ± 16% compared with pretreatment. When expressed as relative values compared with base line, all effects were statistically different from placebo ($P < .05$ in a Dunnett test). Superselective infusion of the compound into the spastic artery did not further dilate the spastic basilar artery, indicating that systemic administration of the compound reached a plateau effect. Such an effect corresponded to about half the maximal

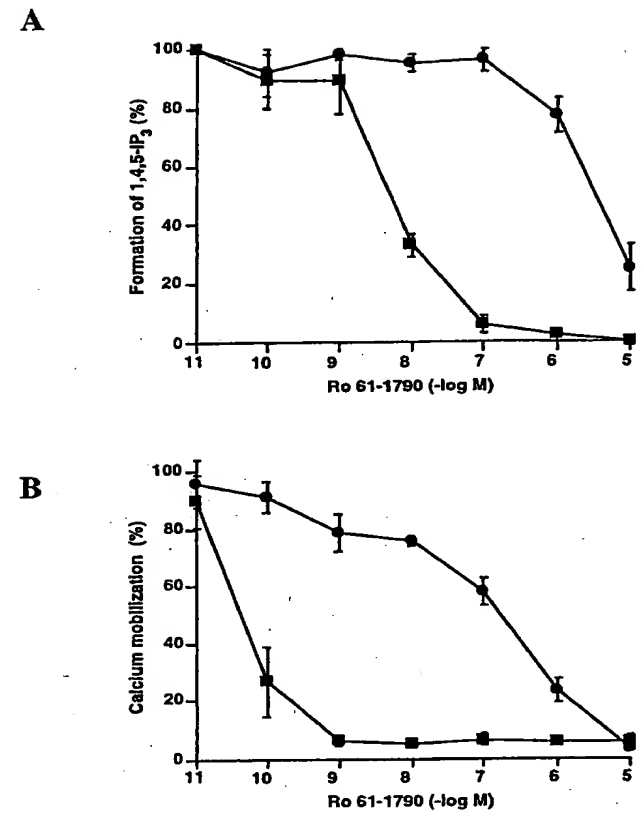

Fig. 3. Concentration-response curves of Ro 61–1790 on (A) the inhibition of ET-1 induced Ins(1,4,5)P_3 formation in COS cells transiently transfected with human ET_A (■) or ET_B (●) receptors. The cells were activated by 100 nM ET-1. Calculated IC_{50} values were 5 nM and 3 μM for the ET_A and ET_B receptor, respectively. B, Inhibition of ET-1-induced Ca^{++} mobilization in HEK 293 cells expressing human ET_A and ET_B receptors. The cells were activated by 100 nM ET-1. The deduced IC_{50} values for ET_A and ET_B are 0.23 ± 0.1 and 422 ± 450 nM, respectively. Values are mean ± S.D. of three independent experiments performed in triplicate.

vasodilatory capacity of these spastic vessels achieved by local infusion of a high dose of papaverine (fig. 6). At the maximal efficacious dose, Ro 61–1790 reached a CSF concentration of 160 nM.

Prevention of cerebral vasospasm. In the placebo group of this prevention study, SAH induced on day 4 an average 40 ± 9% decrease of the cross-sectional area, a vasospasm somewhat less severe than in the reversal study. The low-dose Ro 61–1790 group was not statistically different from placebo (36 ± 10%). However, the high-dose Ro 61–1790 group did not show any vasospasm (3 ± 7%). Papaverine significantly increased the basilar artery in the placebo and low-dose group but not in the high-dose group that was already fully dilated (fig. 7). ET-1 concentration was increased in the CSF after SAH, and Ro 61–1790 only modestly affected plasma and CSF ET-1 levels (table 3).

Pharmac kinetics

The main pharmacokinetic parameters are shown in table 2. The data indicate species differences in the kinetics. Ro 61–1790 exhibited low systemic clearances in both rats and dogs, which represented at most 30% (rats) and 15% (dogs) of the corresponding liver blood flow. In contrast, the systemic clearances in rabbits, cynomolgus and rhesus monkeys were higher, ranging from 15 to 27 ml/min/kg. The volume of distribution corresponded to the extracellular space in rats and in both primate species and was even larger in rabbits, indicating a good tissular penetration of Ro 61–1790. Distribution was limited to the distribution space of albumin in dogs. The half-life for the apparent elimination phase of Ro 61–1790 was <1 hr in all species except rabbits (2.6 hr).

Discussion

The present study describes the biochemical and pharmacological profile of Ro 61–1790. Ro 61–1790 is a new potent, selective and water-soluble ET receptor antagonist able to reverse or prevent cerebral vasospasm in a dog model of SAH.

In vitro, Ro 61–1790 binds with a high affinity (K_i, 0.1–1.7 nM) on membranes expressing human ET_A receptors. The differences in affinity observed for the human ET_A receptors expressed in different systems may be due to variation in the three-dimensional folding that results from different glycosylation or G protein coupling of the receptor. This high affinity binding of Ro 61–1790 on the ET_A receptors is also confirmed in functional assays. The dose-dependent inhibition of ET-1-induced contraction of rat aorta yields to a pA_2 of 9.5 and on ET-1-mediated Ins(1,4,5)P_3 formation and calcium increase in the low nanomolar range. For comparison, compounds such as bosentan, BMS-182,874, PD-156,707, L-754,142, SB-209,670 and A-127,722 exhibit pA_2 values between 7.1 and 9.4 on ET_A-mediated contraction (Battistini and Botting, 1995; Opgenorth *et al.*, 1996). Thus, Ro 61–1790 is one of the most potent compounds described to date for antagonism of the ET-1-induced vasoconstrictor response.

In vivo, potency was assessed in tests such as the big ET-1-induced pressor response in pithed rats. As opposed to ET-1, its precursor big ET-1 induces only a pressor effect (Clozel *et al.*, 1994). Likewise, Ro 61–1790 inhibits the pressor effect with an ID_{50} as low as 0.05 mg/kg, thereby confirming the high *in vivo* potency of this compound.

In addition to *in vitro* and *in vivo* potency, Ro 61–1790 is selective for the ET_A receptor. A selectivity of ~1000-fold for the ET_A receptors is observed for the inhibition of vessel contraction. Ro 61–1790 has a pA_2 value of 6.4 for ET_B receptor-mediated contraction of rat trachea, a value similar to that obtained for bosentan, thereby conferring a 3-order of magnitude selectivity for the ET_A receptor (Clozel *et al.*, 1994).

Ro 61–1790 dose-dependently decreases arterial blood pressure in conscious DOCA-salt hypertensive rats. The DOCA-salt rats instrumented with telemetry offer an optimal opportunity for comparing in conscious rats the relative potencies and durations of action of different ET antagonists. As previously reported, this type of hypertension is associated with an increase in vascular ET-1 concentration (Fujita *et al.*, 1995). In this model, ET-1 makes an important contribution to the maintenance of high blood pressure through the ET_A receptor (Fujita *et al.*, 1995); therefore, this model allows one to study the effects of compounds on endogenously produced ET-1, which is secreted by endothelial cells in a polarized fashion toward the smooth muscle cell layer (Mima *et al.*, 1989). This seems more relevant than animal models in which admin-

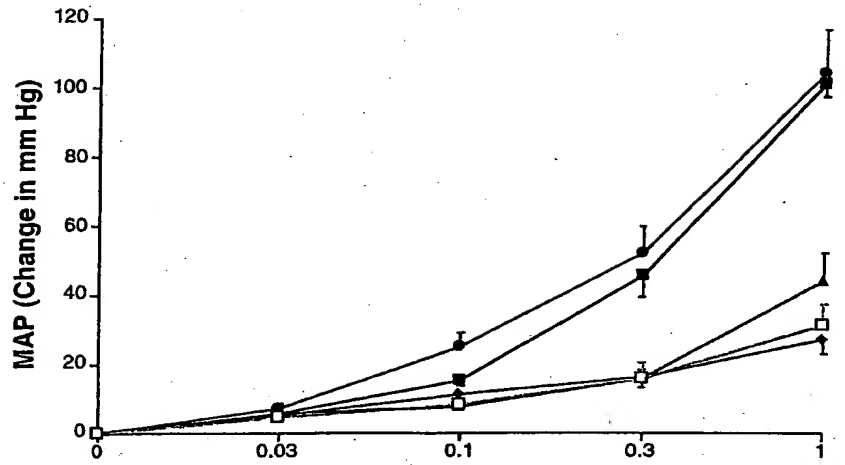

Fig. 4. Effects of Ro 61–1790 (●, 0.01 mg/kg; ▲, 0.1 mg/kg; ◆, 1 mg/kg, □ 3 mg/kg, n = 4 each, or ■, saline, n = 5) on the pressor effect of big ET-1 in pithed rats (ID_{50} = 0.05 mg/kg).

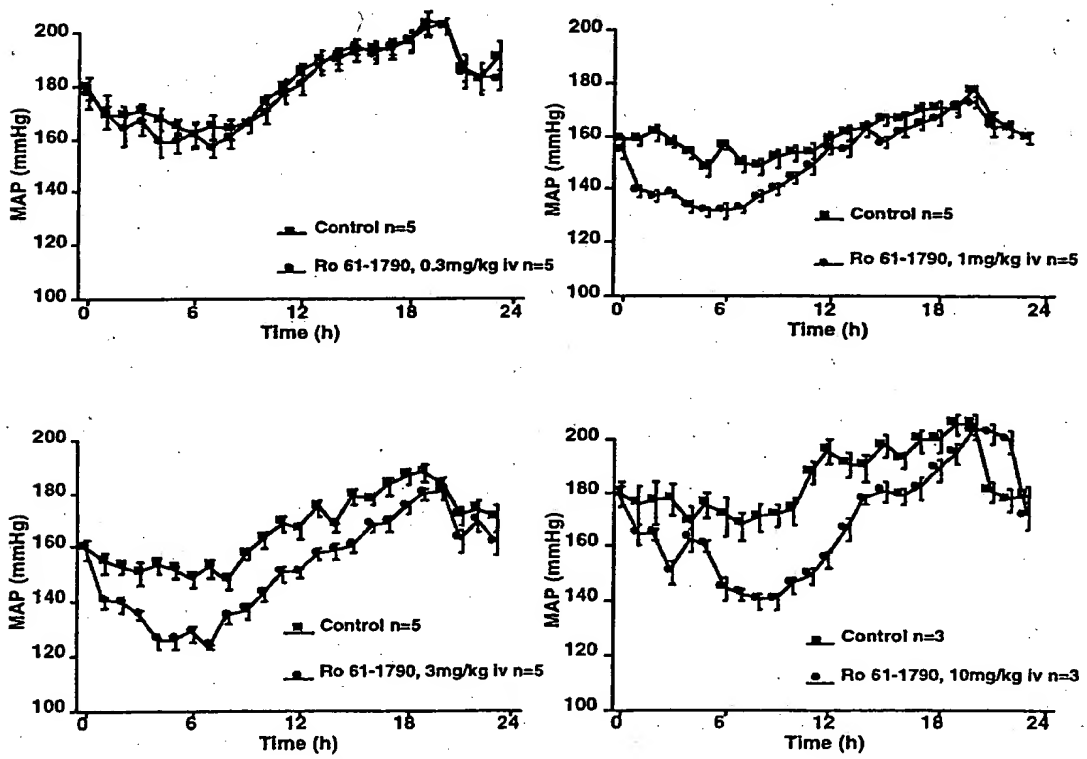

Fig. 5. Dose-dependent decrease of mean arterial pressure (MAP) after single-bolus i.v. administration of Ro 61–1790 in conscious DOCA-salt hypertensive rats.

istration of exogenous ET-1, reaching first the endothelial cell layer, is needed. The short half-life of Ro 61–1790 is in contrast with the long duration of action of the compound as seen in the DOCA-salt rats. This discrepancy could be related to the high affinity of Ro 61–1790 for the ET receptors as well as its distribution to tissue. In this matter, other antihypertensive drugs such as angiotensin-converting enzyme inhibitors showed the same dissociation between their kinetics and dynamic effects (Unger *et al.*, 1981).

The present study shows also that Ro 61–1790 dose-dependently reverses cerebral vasospasm in a canine model of SAH, and this effect reaches a plateau at a dosage of 3 mg/kg/hr.

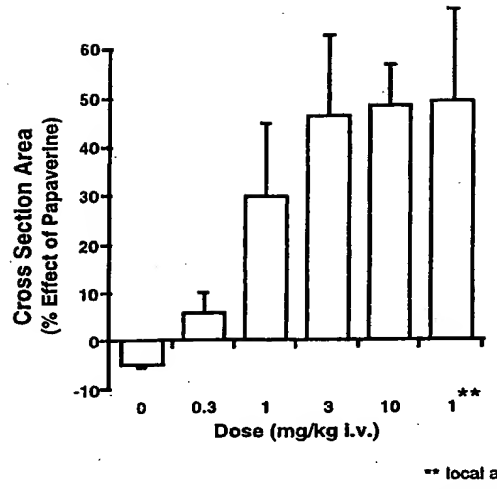

Fig. 6. Dose-dependent reversal of cerebral vasospasm by Ro 61–1790 in SAH dogs (results are expressed as percentage of papaverine effect set at 100%). Results are the mean ± S.E.M. (n = 6). All intravenously treated groups were significantly different from the placebo group ($P < .05$).

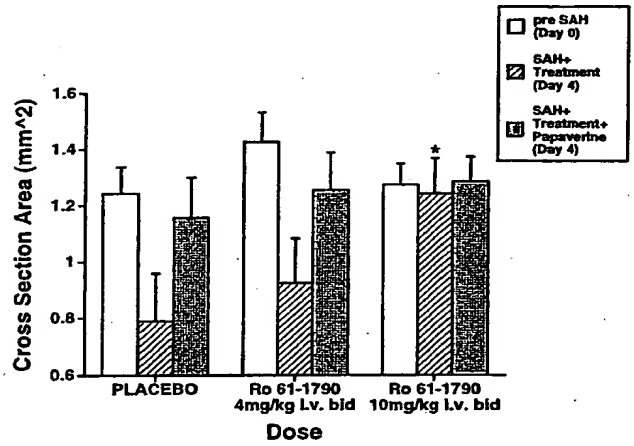

Fig. 7. Prevention of cerebral vasospasm by Ro 61–1790 chronic treatment. Cross-sectional area of the dog basilar arteries (mm^2) are shown before SAH (pre-SAH) and 4 days after the double hemorrhage (SAH). In the high-dose group, no vasospasm was seen on day 4, papaverine did not further vasodilate the basilar arteries. *, $P < .05$ *vs.* placebo.

Noteworthy, the maximal effect of Ro 61–1790 corresponds to half the maximal vasodilation reached by local infusion of a high dose of papaverine, a nonspecific vasodilator. Papaverine is known to be efficacious in human vasospasm, but its local effect is only short acting, and it cannot be administered intravenously (Kassell *et al.*, 1992; Macdonald *et al.*, 1995). In a separate study, high local concentration of Ro 61–1790 achieved by an intrabasilar infusion of 1 mg/kg Ro 61–1790 into the spastic basilar artery was not more efficacious than intravenous administration, implying that the ET-dependent vasospasm represents, at least in dogs, half of the reversible vasospasm.

More importantly, Ro 61–1790 administered at a daily dose of 20 mg/kg prevented the occurrence of vasospasm on day 4 after SAH. Taken together, these data suggest that Ro 61–1790 administered sufficiently early could prevent the occurrence of delayed ischemic deficit due to vasospasm in patients with SAH.

Our data confirm that Ro 61–1790 has a higher potency than bosentan in a model of SAH because the latter reversed cerebral vasospasm in the same model to the same extent but at a 10-fold higher dose (Roux *et al.*, 1995). Our studies also confirm that an intravenous administration of an ET_A antagonist can reverse or prevent cerebral vasospasm without affecting blood pressure, which represents an advantage over nimodipine (Porchet *et al.*, 1995).

Accumulating evidences suggest that a potent ET_A antagonist would be of particular interest in situations of brain cerebral vasospasm in which ET_A seems to play a prominent role. The ET_A subtype is the predominant receptor in brain vascular smooth muscle and endothelial cells and thereby appears to be the most important subtype in the brain vessels (Kawai *et al.*, 1995; Stanimirovic *et al.*, 1994; Vigne *et al.*, 1996; Yu *et al.*, 1995). Topical application of PD 156,707, an ET_A antagonist selectively dilated cortical arterioles in the ischemic penumbra after focal cerebral ischemia and reduced ischemic damage in a cat model of focal cerebral ischemia (Patel *et al.*, 1995, 1996). Interestingly, it was recently shown that BQ 123 and Ro 61–1790, both ET_A-selective antagonists, reversed postischemic hypoperfusion in a gerbil model of global ischemia, whereas bosentan was not active (Yasuma *et al.*, 1997). One hypothesis is that blockade of nitric oxide generation *via* ET_B inhibition in brain microvessels by bosentan would offset the beneficial effect obtained with ET_A blockade.

Pharmacokinetic studies revealed large differences among species. Ro 61–1790 has a low systemic plasma clearance in both rats and dogs, which are at most 30% (rat) and 15% (dog) of the corresponding liver blood flow in these species. In rabbits, cynomolgus and rhesus monkeys, the systemic plasma clearances are close to the liver blood flow values. The volume of distribution corresponds to the distribution volume of serum albumin in dogs and to the extracellular space in rats, as in both primate species. It was even larger in rabbits, indicating good penetration of Ro 61–1790 into tissues in all species except the dog. The half-life for the apparent elimination phase of Ro 61–1790 is <1 hour in various species, including rats, which should be adequate to reach the steady state quickly when infused.

In conclusion, Ro 61–1790 belongs to a new generation of functionalized heteroarylsulfonamido pyrimidine ET antagonists, with high affinity for the ET_A receptor and high aqueous solubility at physiological pH. Ro 61–1790 shows efficacy in two models on which ET_A receptors are known to play a role, namely, the DOCA-salt hypertensive rat and experimental SAH. The combined potency and water solubility as well as the pharmacokinetics of the compound would create the best conditions for a rapid onset of action with no venous toxicity after repetitive parenteral use in patients with SAH.

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TABLE 2
Pharmacokinetic variables of Ro 61-1790

Species	Dose	C_{max}	Systemic plasma clearance	V_{ss}	Apparent $t_{1/2}$ elimination
	mg/kg	*μg/ml*	*ml/min/kg*	*l/kg*	*hr*
Rat	10 (1)	39.8 ± 2.5 (1)	11.7 ± 2.0	0.32 ± 0.10	0.8 ± 0.2
	60 (2)	18.9 ± 5.1 (2)			
Dog	5–6	61.7–76.1 (1)	2.89 ± 0.82	0.11 ± 0.04	0.9 ± 0.2
Rabbit	10	26.8 ± 5.5	26.8 ± 7.3	1.38 ± 0.09	2.6 ± 0.6
Cynomolgus monkey[a]	10	37.8–52.6	15.1–18.8	0.16–0.24	0.5–0.6
Rhesus monkey	10	35.9 ± 12.7	19.0 ± 4.5	0.36 ± 0.26	0.8 ± 0.2

C_{max}, maximal achieved plasma concentration at 5 min after dosing; V_{ss}, volume of distribution at steady state. Data were obtained after single dose intravenous administration (1) or 6-h intravenous infusion (2). Value are mean ± S.D. from 3 to 7 animals.
[a] Individual values, $n = 2$.

TABLE 3
Effects of Ro 61-1790 on plasma and CSF concentrations of ET-1 in dogs with SAH (prevention study)

	Pre-SAH		Day 2		Day 4	
	Plasma	CSF	Plasma	CSF	Plasma	CSF
			pg/ml			
Placebo	7.2 ± 1.1	1.2 ± 0.3	12.2 ± 1.5	4.6 ± 0.9[b]	11.2 ± 1.5	5.4 ± 1.8[b]
Ro 61-1790 (low-dose)	8.6 ± 1.0	2.1 ± 0.9	14 ± 0.7	6.5 ± 0.9[b]	15 ± 1.4[ab]	8.9 ± 2.2[b]
Ro 61-1790 (high-dose)	11.3 ± 1.4	1.0 ± 0.3	17.0 ± 1.6[b]	5.2 ± 0.9[b]	16.0 ± 1.1[ab]	6.8 ± 1.6[b]

Ro 61-1790 was administered 4 and 10 mg/kg twice a day in the low- and high-dose groups, respectively.
[a] $P < .05$ *vs.* placebo.
[b] $P < .05$ *vs.* pre-SAH.

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